

Density-dependent effects on establishment of *Necator americanus* and *Ancylostoma ceylanicum*

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ABSTRACT

The relationships between the number of infective larvae (L3s) to which animals were exposed and the establishment of *A. ceylanicum* and *N. americanus* in hamsters were examined. There was no evidence of density-dependent constraints on the establishment of *A. ceylanicum* in the range 10–1000 larvae, but an experiment in which the range was extended to 1500 L3s gave a significant negative correlation between the magnitude of the infecting dose and percentage establishment. Even so the percentage reduction was relatively small, approximating to 0.89% per 100 larvae administered, and in practice density-dependent constraints on establishment are unlikely to affect experiments with this species in which much lower doses (<250 L3) are generally employed. The range of doses for *N. americanus* L3s was smaller (10–400). Of the four experiments reported, two gave a significant reduction of establishment with increasing dose and two did not. When the data was split into low doses (<100 L3s) and high doses (>100 L3s), falling establishment with increasing dose was only detected in the lower dose range. There was no difference in the establishment when doses of 100 L3s were compared with 250 or 400 L3s. On balance, it was concluded that density-dependent constraints on establishment of *N. americanus* in hamsters were not marked and would have little significant effect on experiments utilizing fewer than 200 L3s (approximately 7.6% reduction between 10 and 200 L3s). These results are discussed in relation to host regulation of hookworm burdens.

KEY WORDS: *Necator americanus*, *Ancylostoma ceylanicum*, hookworms, hamster, density-dependence, establishment, Nematoda

INTRODUCTION

Hookworm infections are among the most widespread of human helminth diseases (SCHAD & WARREN, 1990) and differ from other soil-transmitted nematodes in several aspects of their epidemiology (BUNDY, 1990). In particular, unlike *Ascaris lumbricoides* and *Trichuris trichiura*, worm burdens accumulate with time and the heaviest worm burdens are usually encountered in middle-aged and the elderly inhabitants of affected communities (BUNDY & KEYMER, 1991; BEHNKE, 1987). The stability of age-intensity profiles of communities with hookworms and the rapid re-infection after chemotherapy suggest that hookworm burdens are not easily regulated by the host, acquired immunity playing a minimal role, if any (BEHNKE, 1987, 1991).

The consequence of host-protective regulatory mechanisms acting on hookworm larvae can only be measured indirectly in man through quantification of adult worm burdens after expulsion chemotherapy, through faecal egg counts or through accompanying serum antibody and peripheral blood leukocyte responses. In particular, density-dependent constraints on the biology of hookworms are poorly documented with the notable exception of effects on the fecundity of female worms (SARLES, 1929; KRUPP, 1961). Some of the limitations of studies employing infected human subjects can be overcome in experimental model systems and in this context the adaptation of two species of hookworms (*Necator americanus* and *Ancylostoma ceylanicum*, both infective to man and capable of causing patent infections) for passage through hamsters (SEN, 1972; RAY *et al.*, 1972) has provided an opportunity for comparative studies of aspects of their biology which cannot be readily tackled in man (GARSIDE & BEHNKE, 1989; ROSE & BEHNKE, 1990). Events associated with the establishment, migration and

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development of larvae can be conveniently quantified and compared between these two since both laboratory models involve the same non-human host. In this paper we report experiments which sought to establish whether density-dependent constraints influence the efficiency of establishment of these hookworms in their laboratory host.

MATERIALS AND METHODS

Parasites and hosts

Infective larvae of *N. americanus* were obtained in 1983 from Dr Rajasekariah of CIBA-GEIGY Hindustan Ltd., Bombay, India and the parasite has been maintained since by regular passage through hamsters as described originally by SEN (1972) and (BEHNKE *et al.* (1986a, b). It is important to note here that *N. americanus* will only mature to patency following exposure of neonatal (1–3 day old) hamsters to infection (RAJASEKARIAH *et al.*, 1985). Experiments with *N. americanus* were therefore limited by the need for synchronized mating of female hamsters and by resulting litter sizes. *A. ceylanicum* was also obtained from Dr Rajasekariah and was passaged through adult hamsters using techniques which have been described by GARSIDE & BEHNKE (1989). Worms were recovered by a modified Baerman technique, in which the entire small intestine was suspended in a gauze and incubated for a minimal period of 4 h at 37°C in 50 ml beakers containing Hanks' saline. After incubation the contents of each beaker were emptied into a Petri dish and worms were counted as they were individually picked out with a Pasteur pipette. All the animals used in this work were syngeneic DSN hamsters originally purchased from Intersimian Ltd., Oxford, UK, but now maintained, under conventional conditions with access to food and water *ad libitum*, as a closed breeding colony in the Department of Life Science at Nottingham University.

Statistical analysis of results

The results are presented as group mean values (MWR) \pm standard error (S.E.M.) or as raw data in figures showing correlational relationships between variables. Non-parametric statistical procedures were used to analyse the data sets, because the normal distribution of data could not be assumed (SOKAL & ROHLF, 1969). Correlations between variables were tested by the Spearman Rank Order Correlation Test and the statistic r_s is given, as appropriate.

EXPERIMENTAL DESIGN AND RESULTS

Ancylostoma ceylanicum

Two experiments were carried out to determine whether there is any influence of parasite density on the establishment of larvae after oral infection. In Experiment 1, 35 female hamsters were separated into seven experimental groups of five each and were inoculated orally with 10, 25, 50, 100, 250, 500 or 1000 infective larvae. All the animals were killed seven days post infection (PI) for worm recovery. The mean worm recovery (MWR) is shown in Fig. 1 and the results are expressed as a percentage of the dose administered (percentage establishment) in Fig. 2A. The percentage of the inoculated dose recovered varied from 56% (group given 1000 L3) to 84% (group given 100 L3) but, despite the negative trend for establishment with increasing dose size, the relationship was not statistically significant.

Experiment 2 was carried out to examine in greater detail the relationship between magnitude of dose and percentage establishment at high infection doses. Eighteen male and 5 female hamsters were used and these were arranged as follows: 4 groups of males were given 250 (n=4), 500 (n=4), 750 (n=5) or 1500 (n=5) L3 and one group of females

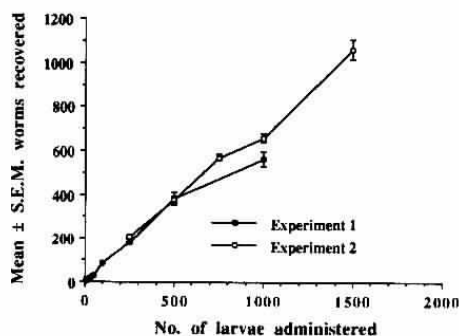


FIG. 1. Relationship between the number of *Ancylostoma ceylanicum* recovered 7 days PI and the size of the infection dose administered.

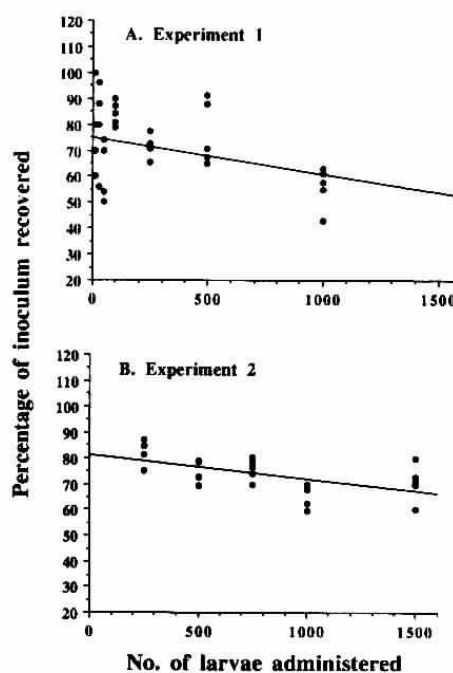


FIG. 2. Relationship between the percentage of the dose of *Ancylostoma ceylanicum* larvae established and the size of the inoculum administered.

Statistical analysis

Experiment 1, $r_s = -0.164$, $n = 35$, $p = 0.35$.

Experiment 2, $r_s = -0.592$, $n = 23$, $p = 0.003$.

received 1000 L3 ($n = 5$). Again all the hamsters were killed for worm recovery 7 days PI. The results are illustrated in Figs 1 and 2B. Percentage establishment varied from 66% (hamsters given 1000 L3s) to 82% (hamsters given 250 L3s) and there was a significant negative correlation between percentage establishment and the number of larvae in the inoculum administered ($p = 0.003$).

Necator americanus

Experiments with *N. americanus* were limited by the availability of litters born on the same day and by the restriction on the maximum infection dose which could be administered, data from earlier unpublished experiments indicating that doses in excess of 400 infective larvae resulted in high mortality within 2 weeks of infection.

Two small scale pilot experiments were first carried out. In Experiment 3 two litters were infected either with 100 ($n = 5$) or 250 ($n = 5$) infective larvae and were killed for worm recovery 11 days later. The percentage of the inoculum recovered was $81.2 \pm 3.2\%$ and $82.4 \pm 0.9\%$ for the groups exposed to low and high doses respectively ($p = \text{not significant}$).

In Experiment 4 three litters were given 10 ($n = 2$), 25 ($n = 3$) or 100 ($n = 4$) L3s and were killed on day 11 PI. Establishment varied from $59.25 \pm 2.56\%$ in animals given 100 L3s, $70.7 \pm 8.1\%$ in the group given 25 L3s to 90% in those exposed to 10 L3s ($r_s = -0.75$, $p = 0.02$).

The final two experiments (Experiments 5 and 6) involved larger numbers of animals, 23 and 53, respectively. In Experiment 5 hamsters were given 10 ($n = 4$), 25 ($n = 6$), 50 ($n = 3$), 100 ($n = 6$) or 250 ($n = 4$) L3s. Experiment 6 extended the range to 400 L3s. The groups were as follows: 25 L3s ($n = 9$), 50 L3s ($n = 14$), 100 L3s ($n = 10$), 250 L3s ($n = 10$) and 400 L3s ($n = 10$). The results are illustrated in Figs 3 and 4. The inoculum of infective larvae used in Experiment 5 was marginally more infective than

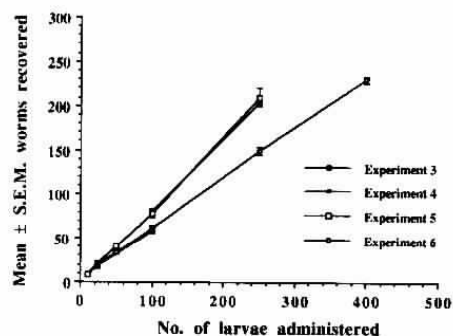


FIG. 3. Relationship between the number of *Necator americanus* recovered 11 days PI and the size of the infection dose administered.

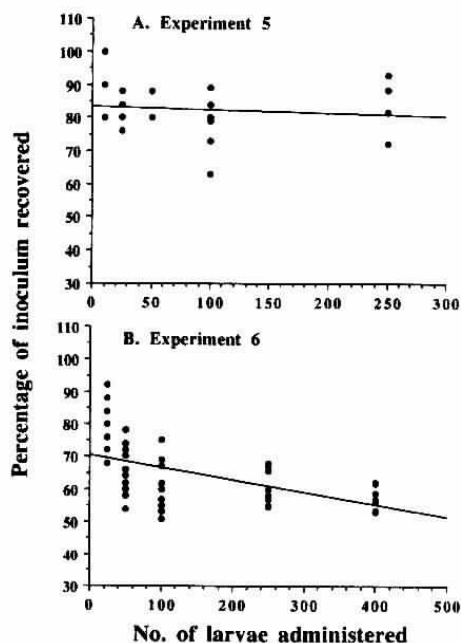


FIG. 4. Relationship between the percentage of the dose of *Necator americanus* larvae established and the size of the inoculum administered.

Statistical analysis

Experiment 5, $r_s = -0.249$, $n = 23$, $p = 0.25$.

Experiment 2, $r_s = -0.68$, $n = 53$, $p < 0.001$.

that in Experiment 6 at low doses with percentage establishments of 82.0% and 78.7% (both at 25 L3s) respectively. Although there was a negative trend for establishment with increasing dose size in both experiments the gradient was less steep in Experiment 5 and was significant only in Experiment 6.

DISCUSSION

Density-dependent constraints on the growth and fecundity of parasites are well established and have been recorded for many different species (KEYMER, 1982; MICHAEL & BUNDY, 1989). However, there is less information on the influence of density of parasites on the establishment and initial survival of infective stages and in this latter context almost no relevant data for hookworm infections in general and for species affecting man in particular. Among the few relevant studies, ONWULIRI *et al.* (1981) reported that the percentage establishment of *A. tubaeforme* in cats decreased from 76.25% in animals given 110 L3s to only 11.2% in those exposed to 2000 L3s. However, their experiment monitored several other aspects of the host-parasite relationship and the animals were killed after 10 weeks of infection. Thus the figures presented do not necessarily reflect establishment but rather survival of worms until 10 weeks PI, a period during which other density-dependent processes such as constraints on feeding at high infection levels and even host immunity may have determined the worm burden surviving to autopsy (see ROSE & BEHNKE, 1990). Establishment of parasites should be quantified as early as possible after exposure to infective larvae to minimize the influence of other variables. As has been pointed out previously (ANDERSON & MAY, 1985), data on establishment of human parasites in their natural host are not obtainable for obvious reasons and since experiments in dogs (which are

susceptible to both *N. americanus* and *A. ceylanicum*) are costly, the results presented in this paper have provided a unique data-set on the relationship between the size of the inoculum administered and the resulting establishment of larvae in hosts which are susceptible to infection.

Although we used hamsters for both species of hookworms, there are important differences between the two model systems which need to be emphasized before evaluation of the results (BEHNKE, 1990). Experiments with *A. ceylanicum* were carried out in mature hamsters infected orally. Percutaneous infections are possible in this species but our experience shows that establishment is greatly inferior to orally administered larvae (GARSIDE & BEHNKE, 1989) and the latter route is therefore the most commonly used method for infecting rodents with this species. We chose day 7 PI for autopsy because unpublished data suggest that recovery of larvae any earlier is prone to greater variation and is often lower because some worms penetrate into the mucosa and possibly venture temporarily into body tissues from which they cannot be recovered efficiently. *N. americanus* larvae were administered percutaneously since this is the only known method for infection with this species (BEHNKE, 1990) and furthermore, in contrast to *A. ceylanicum*, only neonatal (2-day old) hamsters were employed since older hamsters show increased resistance to infection and develop correspondingly lower intestinal worm burdens (RAJASEKARIAH *et al.*, 1985). The earliest time at which the worm burdens could be accurately quantified with this species was day 11 PI, at which time all the larvae have left the lungs and have arrived in the small intestine (BEHNKE *et al.*, 1986a). Although it is possible to estimate worm burdens earlier, as for example in the lungs (BEHNKE *et al.*, 1986b), these represent a migrating population of parasites and our experience shows that worm counts are less accurate than on day 11. It is also pertinent that there is a ceiling on infection doses in excess of 400 L3s in the *N. americanus*/neonatal hamster model since young hamsters do not tolerate worm burdens of a greater magnitude passing through and developing in their lungs (unpublished observations).

The data presented in this paper indicate that no significant influence of size of inoculum on establishment of *A. ceylanicum* was evident at the dose levels which would normally be used in hamsters (<250 L3s). At higher doses a significant reduction in establishment was detected, but even so the gradient of the slope was so shallow that compared with a dose of 10 larvae, a dose of 1000 larvae would result in a reduction of only 8.9%. Thus for all practical purposes with respect to experimentation with *A. ceylanicum* in hamsters no significant reduction in establishment may be anticipated. However, experimenters working with dogs have used far heavier inocula, of the order of 2000 L3s (CARROLL & GROVE, 1984; BEHNKE, 1991), and, by extrapolation from our data, at these doses a percentage reduction in establishment of the order of 17–18% may be anticipated, although the considerable difference in body size between the hamsters and dogs would also have to be considered.

Of the four experiments with *N. americanus*, two found no density-dependent influence on establishment and two (Experiments 4 and 6) detected a significant effect. Experiment 2 found virtually no difference in % recovery between groups given 100 and 250 L3s and the data in Fig. 4 shows that Experiments 5 and 6 concur, when just these infection levels are considered. When data for doses of <100 L3s were excluded no significant differences were found in percentage establishment between 100 and either 250 or 400 L3s in any of the relevant experiments (4, 5 and 6). However, when the range 10 to 100 L3s was examined, Experiment 4 indicated that there was a significant effect. Again, it is this range in Experiment 6 (Fig. 4B) which gives the steepest gradient (not illustrated separately), and closer examination of mean percentage establishment shows that the two data sets are very similar at doses which correspond.

Thus, following exposure to 25 and 100 L3s in Experiment 4, 70.7 and 59.25% of the inoculum was recovered and in Experiment 6, 79.6 and 61.6%. In contrast, values for these doses in Experiment 5 were 82 and 78% respectively. Surprisingly, therefore, it would appear as though density-dependent constraints exist in the lower range of inocula and it may be that this is partly artifactual stemming from the greater influence on the overall mean and percentage recovery of failing to find 1–2 worms. At higher infection levels, a few undetected worms would have a proportionally smaller influence on the result. Comparable problems relating to density-dependent effects on worm fecundity have been discussed in the literature (KEYMER & SLATER, 1987). Nevertheless, the overall density-dependent effect on establishment, within the range normally used for experimentation (<200) was not marked (by extrapolation from Experiment 6 a reduction of 7.6%).

Finally, our results have provided, for the first time, quantitative data on density-dependent effects on the establishment of hookworm species infective to man. Comparable data are unlikely ever to be obtained for human infections and therefore our results make a pertinent contribution to the understanding of the processes which regulate hookworms *in vivo*. Although in some respects not ideal hosts, the hamsters used in this study were susceptible to both species and were capable of sustaining chronic patent infections (BEHNKE, 1990). Moreover, as abnormal hosts, regulatory mechanisms might have been expected to operate in hamsters with greater efficiency than in the normal definitive hosts. The conclusion that there were only minimal density-dependent constraints on establishment of hookworm larvae in hamsters suggests that regulation of hookworms at this level is unlikely to be of significance to the population dynamics of these parasites in their natural hosts.

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REFERENCES

- ANDERSON, R. M. & MAY, R. M. (1985) Helminth infections of humans: mathematical models, population dynamics and control. *Advances in Parasitology*, **24**, 1–101.
- BEHNKE, J. M. (1987) Do hookworms elicit protective immunity in man? *Parasitology Today*, **3**, 200–206.
- BEHNKE, J. M. (1990) Laboratory animal models. In: *Hookworm Disease. Current Status and New Directions*. pp. 105–128 (editors, G. A. Schad and K. S. Warren). Taylor and Francis: London, UK.
- BEHNKE, J. M. (1991) Immunology. In: *Human Parasitic Diseases, Volume 4, Hookworm Infections*. pp. 93–155 (editors, H. M. Gilles and P. A. J. Ball). Elsevier: Amsterdam.
- BEHNKE, J. M., PAUL, V. & RAJASEKARIAH, G. R. (1986a) The growth and migration of *Necator americanus* following infection of neonatal hamsters. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80**, 146–149.
- BEHNKE, J. M., WELLS, C. & BROWN, J. (1986b) An improved technique for experimental infections with skin penetrating nematode larvae (*Necator americanus*). *International Journal for Parasitology*, **16**, 461–464.
- BUNDY, D. A. P. (1990) Is the hookworm just another geohelminth? In: *Hookworm Disease. Current Status and New Directions*. pp. 147–164. (editors, G. A. Schad and K. S. Warren). Taylor and Francis: London, UK.
- BUNDY, D. A. P. & KEYMER, A. E. (1991) The epidemiology of hookworm infection. In: *Human Parasitic Diseases, Volume 4, Hookworm Infections*. pp. 157–178. (editors, H. M. Gilles and P. A. J. Ball). Elsevier: Amsterdam.
- CARROLL, S. M. & GROVE, D. I. (1984) Parasitological, hematologic and immunologic responses in acute and chronic infections of dogs with *Ancylostoma ceylanicum*: a model of human hookworm infection. *Journal of Infectious Diseases*, **150**, 284–294.

- GARSIDE, P. & BEHNKE, J. M. (1989) *Ancylostoma ceylanicum*: observations on host-parasite relationship during primary infection. *Parasitology*, **98**, 283–289.
- KEYMER, A. (1982) Density-dependent mechanisms in the regulation of intestinal helminth populations. *Parasitology*, **84**, 573–587.
- KEYMER, A. & SLATER, A. F. G. (1987). Helminth fecundity: density dependence or statistical illusion? *Parasitology Today*, **3**, 56–58.
- KRUPP, I. M. (1961) Effects of crowding and of superinfection on habitat selection and egg production in *Ancylostoma caninum*. *Journal of Parasitology*, **47**, 957–961.
- MICHAEL, E. & BUNDY, D. A. P. (1989) Density dependence in establishment, growth and worm fecundity in intestinal helminthiasis: the population biology of *Trichuris muris* (Nematoda) infection in CBA/Ca mice. *Parasitology*, **98**, 451–458.
- ONWULIRI, C. O. E., NWOSU, A. B. C. & ANYA, A. O. (1981) Experimental *Ancylostoma tubaeforme* infection of cats: changes in blood values and worm burden in relation to single infections of varying size. *Zeitschrift für Parasitenkunde*, **64**, 149–155.
- RAJASEKARIAH, G. R., DEB, B. N., DHAGE, K. R. & BOSE, S. (1985) Site of resistance to *Necator americanus* in hamsters. *Acta Tropica*, **42**, 333–340.
- RAY, D. K., BHOPALE, K. K. & SHRIVASTAVA, V. B. (1972) Migration and growth of *Ancylostoma ceylanicum* in golden hamsters. *Journal of Helminthology*, **46**, 357–362.
- ROSE, R. A. & BEHNKE, J. M. (1990) *Necator americanus* in the DSN hamster: density dependent expulsion of adult worms during primary infection. *Parasitology*, **100**, 469–478.
- SARLES, M. P. (1929) The effect of age and size of infestation on the egg production of the dog hookworm, *Ancylostoma caninum*. *American Journal of Hygiene*, **10**, 658–666.
- SCHAD, G. A. & WARREN, K. S. (editors) (1990) *Hookworm Disease: Current Status and New Directions*. Taylor & Francis: London.
- SEN, H. G. (1972) *Necator americanus*: behaviour in hamsters. *Experimental Parasitology*, **32**, 26–32.
- SOKAL, R. R. & ROHLF, F. J. (1969) *Biometry*. Freeman: San Francisco.

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